

Isolation and Characterization of a Kunitz-Type Trypsin Inhibitor with Antiproliferative Activity from *Gymnocladus Chinensis* (Yunnan Bean) Seeds

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Abstract A 20-kDa Kunitz-type trypsin inhibitor was isolated from *Gymnocladus chinensis* (Yunnan bean) seeds. The isolation procedure involved ion exchange chromatography on diethylaminoethyl cellulose (DEAE-cellulose), affinity chromatography on Affi-gel blue gel, ion exchange chromatography on sulfopropyl sepharose (SP-sepharose), and gel filtration by FPLC on Superdex 75. The trypsin inhibitor was adsorbed on DEAE-cellulose, unadsorbed on Affi-gel blue gel, and adsorbed on SP-Sepharose. It dose-dependently inhibited trypsin with an IC_{50} value of 0.4 μ M. Dithiothreitol reduced its trypsin inhibitory activity, suggesting that an intact disulfide bond is indispensable to the activity. It suppressed [methyl- 3 H] thymidine incorporation by leukemia L1210 cells and lymphoma MBL2 cells with an IC_{50} value of 4.7 and 9.4 μ M, respectively. There was no effect on human immunodeficiency virus⁴-1 reverse transcriptase activity and fungal growth when the trypsin inhibitor was tested up to 100 μ M.

Keywords Isolation · Characterization · Trypsin inhibitor · Antiproliferative activity · *Gymnocladus chinensis* seeds

Abbreviations

DEAE-cellulose	Diethylaminoethyl cellulose
SP-sepharose	Sulfopropyl sepharose
FPLC	Fast protein liquid chromatography
HIV	Human immunodeficiency virus
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
HPLC	High performance liquid chromatography
DTT	Dithiothreitol
DMEM	Dulbecco's modified eagle medium
FBS	Fetal bovine serum
RT	Reverse transcriptase
ELISA	Enzyme-linked immunosorbent assay
IPTG	Isopropyl- β -d-thiogalactopyranoside
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
EDTA	Ethylenediaminetetraacetic acid
CoV	Coronavirus
SARS	Severe acute respiratory syndromes
IC_{50}	Half maximal (50%) inhibitory concentration

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1 Introduction

Legumes produce a diversity of proteins including protease inhibitors, α -amylase inhibitors, arcelins, lectins, and anti-fungal proteins [2, 4, 6, 7, 9, 12–14, 16, 17, 19, 20, 27–29, 31, 34]. Bowman-Birk trypsin inhibitors and Kuntz-type inhibitors with a molecular mass of about 8 and 20 kDa,

respectively, can be isolated from legumes [6, 8, 12, 19, 26, 28].

Legume protease inhibitors have a diversity of functions. On the one hand, some are potent inhibitors to insects. They reduce protein digestion in insects and consequently growth is impaired [15, 26]. Insects become weak and finally succumb. For instance, growth and development in the cowpea weevil (*Callosobruchus maculatus*) and tomato moth (*Lacanobia oleracea*) are adversely affected by soyacystatin and cowpea trypsin inhibitor, respectively [2]. The trypsin inhibitors from broad beans exhibit antifungal activity toward *mycosphaerella arachidicola* [37, 38]. Acacia plumose trypsin inhibitor inhibits the fungi *Aspergillus niger*, *Colletotrichum sp* and *Thielaviopsis paradoxa* [24]. Similarly, wild soybean trypsin inhibitor inhibits *Aspergillum flavus* [39]. On the other hand, Derris trifoliolate trypsin inhibitor exhibits antimalarial activity [3]. Soybean Kunitz trypsin inhibitor exhibits a strong inhibitory effect on migration of human umbilical vein endothelial cells and tubulogenesis in fibrin matrix without exerting toxicity [30]. Bowman-Birk protease inhibitors suppress N-nitrosomethylbenzylamine-induced esophageal carcinogenesis [32] and Kunitz protease inhibitors reduce ovarian cancer cell invasion by inhibiting upregulation of urokinase [18]. Thus these proteins may be useful in agriculture in which they can protect economically important crops from phytophagous insects, and in medicine as anticancer therapeutics.

Gymnocladus chinensis is widely distributed in South China and can be cultivated. It has been used in treatment of furunculosis, soreness and swelling in traditional Chinese medicine for a long time. However, few biological components were isolated. It was thus examined for the presence of antifungal proteins or peptides [35]. In this study, a trypsin inhibitor was isolated from the Yunnan bean. It was tested for antiproliferative activity against tumor cells and inhibitory activity against human immunodeficiency virus (HIV)-1 reverse transcriptase (RT).

2 Materials and Methods

2.1 Isolation of Trypsin Inhibitor

A water extract of the Yunnan beans (250 g) was made by homogenizing them in distilled water (3 ml/g). The homogenate was then centrifuged (14000 g for 25 min at 4 °C). The supernatant was collected and loaded on a 5 × 20 cm column of diethylaminoethyl cellulose (DEAE-cellulose) (Sigma) in 10 mM Tris-HCl buffer (pH 7.4). Following removal of unadsorbed proteins (fraction D1), the column was eluted sequentially with 0.2 M NaCl and 1 M NaCl in the Tris-HCl buffer. Fraction D2 eluted with

0.2 M NaCl was dialyzed and then chromatographed on a 5 × 15 cm column of Affi-gel blue gel (Bio-Rad) in 10 mM Tris HCl buffer (pH 7.4). The unadsorbed proteins (fraction B1) were dialyzed against 10 mM NH₄OAc buffer (pH 5) and chromatographed on a column of sulfo-propyl sepharose (SP-sepharose) (GE Healthcare). After elution of unadsorbed proteins (fraction SP1), the column was eluted with a 0-1 M NaCl concentration gradient in the NH₄OAc buffer. The second adsorbed fraction (SP3) was then subjected to gel filtration on a Superdex 75 HR 10/30 column (GE Healthcare) in 0.2 M NH₄HCO₃ buffer (pH 8.5). The second absorbance (SU2) peak represented purified trypsin inhibitor.

2.2 Assay for Trypsin Inhibitory Activity

The assay for trypsin inhibitory activity was carried out by addition of the sample to be tested (20 µl) to 160 µl of a 1% casein solution in 0.1 M Tris-HCl buffer (pH 7.4). Trypsin (20 µl of a 0.5 mg/ml solution) was then added and the mixture was incubated at 37 °C for 15 min before 0.4 ml 5% trichloroacetic acid was added to terminate the reaction. After centrifugation the absorbance of the supernatant, which reflects the amount of casein fragments, was measured at 280 nm [38].

2.3 Electrophoresis, Molecular Mass Determination, and N-Terminal Sequence Analysis

The purified protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for molecular mass determination [21]. Gel filtration on an FPLC-Superdex 75 column, which had been calibrated with molecular mass markers (GE Healthcare), was conducted to determine the molecular mass of the protein. The markers included blue dextran (to determine void volume), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa). The N-terminal sequence of the protein was determined by using a Hewlett-Packard HP G1000A Edman degradation unit and an HP 1000 HPLC System [21].

2.4 Effect of Dithiothreitol (DTT) on Trypsin Inhibitory Activity

The isolated trypsin inhibitor (2.5 µM) was incubated with DTT at the final concentration 2.5, 10 and 40 mM for 5, 20 and 80 min at 37 °C. Soybean trypsin inhibitor (Sigma, 2.5 µM) and PBS were similarly treated as positive control and negative control, respectively. The reaction was terminated by adding iodoacetamide at twice the amount of thiol functions at each DTT concentration. The remaining trypsin inhibitory activity was measured at pH 7.4 as

described above in assay for trypsin inhibitory activity. The highest iodoacetamide concentration used in the test was devoid of any effect on the activity of trypsin and the trypsin inhibitory activity of isolated trypsin inhibitor and soybean trypsin inhibitor [1].

2.5 Assay for Antiproliferative Activity toward Leukemia Cells and Lymphoma Cells

The antiproliferative activity of the purified protein was determined as follows. The cell lines L1210 (leukemia) and MBL2 (lymphoma) were purchased from American Type Culture Collection. The cell lines were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 mg/l streptomycin and 100 IU/ml penicillin at 37 °C in a humidified atmosphere of 5% CO₂. Cells (1×10^4) in their exponential growth phase were seeded into each well of a 96-well culture plate (Nunc, Denmark) and incubated for 3 h before addition of the protein. Incubation was carried out for another 48 h. Radioactive precursor, 1 μ Ci, ([³H-methyl]-thymidine, from GE Healthcare) was then added to each well and incubated for 6 h. The cultures were then harvested by a cell harvester. The incorporated radioactivity was determined by liquid scintillation counting [35]. A peptide from Nepalese large red beans [25] and PBS were used as positive control and negative control, respectively.

2.6 Assay for HIV-1 Reverse Transcriptase Inhibitory Activity

The assay for HIV-1 RT inhibitory activity was carried out in view of the report that trypsin inhibitors manifest this activity [33, 36]. It was conducted according to instructions supplied with the assay kit from Boehringer Mannheim (Germany). The assay takes advantage of the ability of RT to synthesize DNA, starting from the template/primer hybrid poly (A) oligo (dT) 15. The digoxigenin- and biotin-labeled nucleotides in an optimized ratio are incorporated into one of the same DNA molecule, which is freshly synthesized by the RT. The detection and quantification of synthesized DNA as a parameter for RT activity follows a sandwich enzyme-linked immunosorbent assay (ELISA) protocol. Biotin-labeled DNA binds to the surface of microtiter plate modules that have been precoated with streptavidin. In the next step, an antibody to digoxigenin, conjugated to peroxidase, binds to the digoxigenin-labeled DNA. In the final step, the peroxidase substrate is added. The peroxidase enzyme catalyzes the cleavage of the substrate, producing a colored reaction product. The absorbance of the samples at 405 nm can be determined using a microtiter plate (ELISA) reader and is directly

correlated to the level of RT activity. A fixed amount (4–6 ng) of recombinant HIV-1 RT was used. The inhibitory activity of the protein was calculated as percent inhibition as compared to a control without the protein. The protein control employed was the antifungal protein gymnin [35].

2.7 Assay of Ability to Inhibit HIV-1 Integrase

A non-radioactive ELISA-based HIV-1 integrase assay was performed according to the DNA-coated plate method. In this study, 1 μ g of SmaI-linearized pBluescript SK was coated onto each well in the presence of 2 M NaCl as target DNA. The donor DNA was prepared by annealing VU5BR (5'-biotin-GTGTGGAAAATCTCTA-GCAGT-3') and VU5 (5'-ACTGCTAGAGATTTTCCACAC-3') in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and 0.1 M NaCl at 80 °C followed by 30 min at room temperature. Integrase reaction was performed in 20 mM HEPES (pH 7.5) containing 10 mM MnCl₂, 30 mM NaCl, 10 mM dithiothreitol and 0.05% Nonidet-P40 (Sigma). After the integrase reaction, the biotinylated DNA immobilized on the wells was detected by incubation with streptavidin-conjugated alkaline phosphatase (Boehringer-Mannheim, Mannheim, Germany), followed by colorimetric detection with 1 mg/ml p-nitrophenyl phosphate in 10% diethanolamine buffer (pH 9.8) containing 0.5 mM MgCl₂. The absorbance due to the alkaline phosphatase reaction was measured at 415 nm. The ribosome inactivating protein trichosanthin was used as a positive control [23].

2.8 Screening for Inhibitory Effect on SARS Coronavirus Protease

The activity of SARS coronavirus (CoV) protease was indicated by a cleavage of designed substrate which was composed of two proteins linked by a cleavage site for SARS CoV protease. The reaction was performed in a mixture containing 5 μ M SARS CoV protease, 5 μ M sample, 20 μ M substrate and buffer [20 mM Tris-HCl (pH 7.5), 20 mM NaCl and 10 mM beta-mercaptoethanol] for 40 min at 37 °C. After 40 min, the reaction was stopped by heating at 100 °C for 2 min. Then the reaction mixture was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). If SARS CoV protease is inhibited by the test sample, there is only one band, which is the intact substrate, shown in SDS-PAGE [23].

2.9 Assay of Antifungal Activity

This assay was conducted in view of the report on antifungal activity of some trypsin inhibitors [37]. The assay for antifungal activity toward *Mycosphaerella arachidicola*

and *Fusarium oxysporum* was carried out in 100 × 15 mm petri plates containing 10 ml of potato dextrose agar. After the mycelial colony had developed, sterile blank paper disks (0.625 cm in diameter) were placed at a distance of 0.5 cm away from the rim of the mycelial colony. An aliquot (15 µl) of the isolated trypsin inhibitor was added to a disk. The plates were incubated at 23 °C for 72 h until mycelial growth had enveloped the disks containing the control and had formed crescents of inhibition around disks containing samples with antifungal activity. The protein control used was gymnin [35].

3 Results

3.1 Purification of Trypsin Inhibitor

Upon ion exchange chromatography on DEAE-cellulose, the Yunnan bean extract was separated into three fractions of approximately equal size, an unadsorbed fraction D1 and two adsorbed fractions D2 and D3. Trypsin inhibitory activity was located in fraction D2. When fraction D2 was subjected to affinity chromatography on Affi-gel blue gel, the activity was recovered in the unadsorbed and larger fraction B1 (Table 1). Upon ion exchange chromatography on SP-sepharose, fraction B1 was resolved into a small unadsorbed fraction SP1, two large adsorbed fractions (SP2 and SP3) of approximately equal size and a small strongly adsorbed fraction SP4 (Fig. 1A). Activity resided in fraction SP3. Further purification of SP3 on Superdex 75 yielded two fractions, SU1 and SU2 of approximately equal size (Fig. 1B). Only fraction SU2 exhibited trypsin inhibitory activity. The yields of the various chromatographic fractions are presented in Table 1. SU2 exhibited a molecular mass of 20 kDa in both SDS-PAGE (Fig. 2) and gel filtration (Fig. 1B). The N-terminal sequence of the trypsin inhibitor is shown in Table 2. It bears some resemblance to other leguminous trypsin inhibitors.

3.2 Characterization of Isolated Trypsin Inhibitor

DTT treatment lowered the trypsin inhibiting activity in a dose- and time-dependent manner (Table 3). When the isolated trypsin inhibitor (2.5 µM) was incubated with DTT at the concentration 2.5 mM for 5 min, the inhibition of trypsin inhibitory activity was 4%. As the incubation time was lengthened, the inhibition of trypsin inhibitory activity became more conspicuous. After incubation for 80 min, the trypsin inhibitory activity was inhibited by 42%. Moreover, the extent of inhibition of trypsin inhibitory activity increased with the concentration of DTT. When the DTT concentration reached 40 mM, the trypsin inhibitory activity was inhibited by about 70%. The IC₅₀ of

Table 1 Yields and trypsin inhibitory activities of various chromatographic fractions (from 250 g dry seeds)

Fraction	Yields (mg)	IC ₅₀ (mg/ml)	Fraction	Yields (mg)	IC ₅₀ (mg/ml)
D1	1362	–	SP2	115	–
D2	1146	61.5	SP3	132.1	13
D3	1180	–	SP4	106.5	–
B1	593.5	41.9	SU1	39.7	–
B2	208.7	–	SU2	52.8	7.2
SP1	72.4	–			

Trypsin inhibitor-enriched fractions are highlighted in boldface

– Negligible or undetectable trypsin inhibitory activity

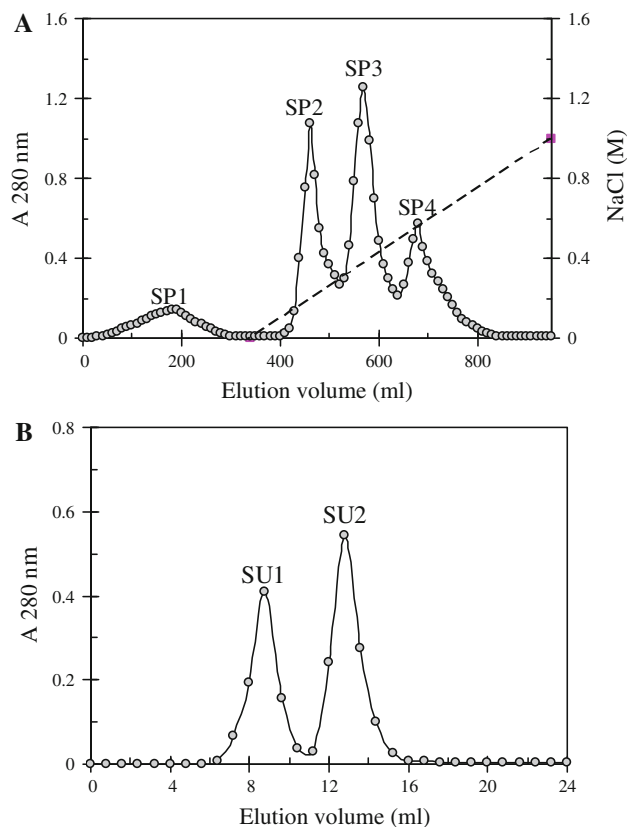


Fig. 1 Purification of *Gymnocladus chinensis* trypsin inhibitor by chromatography. **a** Ion exchange chromatography on SP-sepharose column (2.5 × 20 cm). Sample: fraction of Yunnan bean extract that was adsorbed on DEAE-cellulose and eluted with 0.2 M NaCl, and subsequently unadsorbed on Affi-gel blue gel. Starting buffer: 10 mM NH₄OAc buffer (pH 5). Dotted line indicates 0–1 M NaCl gradient in 10 mM NH₄OAc buffer (pH 5) used to elute adsorbed proteins. Trypsin inhibitory activity was detected only in fraction SP3. **b** Gel filtration by fast protein liquid chromatography on a Superdex 75 HR10/30 column using an AKTA Purifier (GE Healthcare). Sample: fraction SP3 from SP-sepharose column. Buffer: 0.2 M NH₄HCO₃ buffer (pH 8.5). Flow rate: 0.4 ml/min. Fraction size: 0.8 ml. Trypsin inhibitory activity was found exclusively in fraction SU2

Fig. 2 SDS–PAGE results of trypsin inhibitor (7 μ g TI, which was fraction SU2) and molecular mass marker (*right lane*). Molecular mass of trypsin inhibitor (*left lane*) was 20 kDa

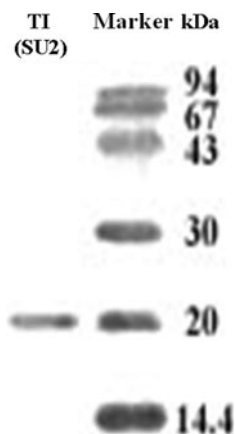


Table 2 Comparison of N-terminal sequences of trypsin inhibitors from *Gymnocladus chinensis* (GCTI) and other leguminous species

Trypsin inhibitor	Amino acid sequence
GCTI (1–33)	<u>K</u> S <u>G</u> H <u>R</u> H <u>E</u> S <u>T</u> D <u>E</u> P <u>S</u> E <u>S</u> K <u>K</u> A <u>D</u> D <u>D</u> H <u>C</u> A <u>C</u> T <u>K</u> <u>S</u> I <u>P</u> P <u>Q</u> Q
VUTI ^a (79–111)	T <u>N</u> H <u>H</u> D <u>S</u> D <u>S</u> D <u>E</u> P <u>S</u> E <u>S</u> E <u>S</u> E <u>P</u> C <u>C</u> D <u>S</u> C <u>I</u> C <u>S</u> K <u>S</u> I <u>P</u> P <u>Q</u> C
VRTI ^b (26–58)	L <u>R</u> S <u>H</u> H <u>H</u> D <u>S</u> D <u>E</u> P <u>S</u> E <u>S</u> E <u>S</u> E <u>P</u> C <u>C</u> D <u>S</u> C <u>R</u> C <u>T</u> K <u>S</u> I <u>P</u> P <u>Q</u> C
PVTI ^c (38–70)	Q <u>L</u> L <u>S</u> N <u>G</u> D <u>A</u> G <u>Y</u> S <u>I</u> K <u>S</u> T <u>T</u> T <u>A</u> C <u>C</u> D <u>S</u> C <u>I</u> C <u>T</u> K <u>S</u> I <u>P</u> P <u>Q</u> C
SBTI ^d (129–162)	<u>K</u> I <u>G</u> E <u>N</u> K <u>D</u> A <u>M</u> D <u>G</u> W <u>F</u> R <u>L</u> E <u>R</u> V <u>S</u> D <u>E</u> F <u>N</u> N <u>Y</u> K <u>L</u> V <u>F</u> C <u>P</u> Q <u>Q</u>

^a Trypsin inhibitor from *Vigna unguiculata*

^b Trypsin inhibitor from *Vigna radiata* var. *radiata* (mung bean)

^c Trypsin inhibitor from *Phaseolus vulgaris*

^d Trypsin inhibitor from soybean seeds

Amino acids identical to those of GCTI are underscored

DTT was 0.5 μ M (Table 3). There was no inhibition on HIV-1 RT HIV-1 integrase and SARS coronavirus when the trypsin inhibitor was tested at various concentrations up to 100 μ M (data not shown). The IC₅₀ values of inhibitory effects of the trypsin inhibitor on L1210 cells and MBL2 cells were, respectively, 4.7 and 9.4 μ M (Table 4).

Table 3 Inhibition rate (%) of dithiothreitol (DTT) on the trypsin inhibitory activity of *Gymnocladus chinensis* trypsin inhibitor and soybean trypsin inhibitor after incubation at 37 °C for different durations

Incubation time (min)	% inhibition of trypsin inhibitory activity			
	Isolated trypsin inhibitor			Soybean trypsin inhibitor
	2.5 mM DTT	10 mM DTT	40 mM DTT	2.5 mM DTT
5	4.0 \pm 0.5 ^a	8.4 \pm 1.0 ^b	13.6 \pm 1.1 ^c	78.2 \pm 5.7 ^j
20	15.9 \pm 1.3 ^d	22.7 \pm 1.9 ^e	37.4 \pm 2.5 ^f	93.7 \pm 5.0 ^k
80	42.1 \pm 3.6 ^g	50.5 \pm 4.3 ^h	68.3 \pm 5.2 ⁱ	96.4 \pm 4.3 ^l

Results are presented as mean \pm SD (n = 3). Different superscripts (e.g. a, b, c) in the same row indicate statistically significant difference ($p < 0.05$) among the extents of inhibition brought about by different DTT concentrations at the same time point, when the data are analyzed by analysis of variance followed by Duncan's multiple range test. PBS was similarly treated as negative control

Inhibition rate (%) of isolated or soybean trypsin inhibitor at x mM DTT = (Trypsin inhibitor activity of isolated or soybean trypsin inhibitor – trypsin inhibitory activity of isolated or soybean trypsin inhibitor in present of x mM DTT) \div trypsin inhibitory activity of isolated or soybean trypsin inhibitor \times 100%

4 Discussion

The trypsin inhibitor from *Gymnocladus chinensis* seeds exhibits a certain extent of similarity in N-terminal sequence to other leguminous species including *Vigna radiata*, *Vigna unguiculata*, *Pisum sativum* and *Phaseolus vulgaris*. Gymnin, an antifungal protein from the same bean, is unadsorbed on DEAE-cellulose and adsorbed on Affi-gel blue gel [35] in contrast to the homologous trypsin inhibitor which is adsorbed on the DEAE-ion exchanger and unadsorbed on the blue gel. A comparison of the trypsin inhibitor with a Kunitz inhibitor isolated from Korean large black soybeans [10] revealed that both of them are unadsorbed on the blue gel. On the other hand, some trypsin inhibitors are antifungal proteins [37, 38]. The purification procedure appears to be efficient in separating antifungal proteins from trypsin inhibitors. The molecular mass and N-terminal sequence of trypsin inhibitor indicate that it is a Kunitz-type trypsin inhibitor.

The reducing agent dithiothreitol reduces the trypsin inhibiting activity of the isolated trypsin inhibitor in a dose- and time-dependent manner. Dithiothreitol is known to reduce disulfide bond to sulfhydryl group. Thus the reduction in trypsin inhibitory activity after DTT treatment indicates that reduction of disulfide bonds results in diminution of trypsin inhibitory activity. This finding supports the contention that the intact disulfide bond is crucial to its trypsin inhibitory activity, reminiscent of the observation on papaya trypsin inhibitor [1].

The isolated trypsin inhibitor inhibits proliferation of L1210 cells and MBL2 cells. In this connection other trypsin inhibitors have also been shown to have antiproliferative activity toward cancer cell lines [22], trigger apoptosis in tumor cells [11], repress carcinogen-induced tumorigenesis [32] and inhibit invasion of tumor cells [18]. However, not all trypsin inhibitors exert a similar action. Lily bulb trypsin inhibitor [40] and trypsin inhibitor from *Vigna mungo* seeds are devoid of antiproliferative activity [5].

Table 4 Inhibition rate (%) of *Gymnocladus chinensis* trypsin inhibitor on leukemia L1210 cells and lymphoma MBL2 cells. Results are presented as mean \pm SD (n = 3)

A		
Dose (μ M)	% Inhibition of <i>Gymnocladus chinensis</i> trypsin inhibitory activity	
	L1210 cells	MBL2 cells
20	92.8 \pm 6.7 ^a	76.9 \pm 7.0 ^g
10	74.5 \pm 6.1 ^b	51.9 \pm 4.8 ^h
5	52.7 \pm 5.4 ^c	29.1 \pm 3.2 ⁱ
2.5	20.6 \pm 3.1 ^d	17.4 \pm 2.1 ^j
IC ₅₀ (μ M)	4.7	9.4
B		
Dose (μ M)	% Inhibition of peptide from Nepalese large red beans activity	
	L1210 cells	MBL2 cells
60	95.6 \pm 7.2 ^a	51.5 \pm 4.8 ^g
20	92.7 \pm 7.6 ^b	28.9 \pm 3.0 ^h
10	81.1 \pm 7.4 ^c	14.2 \pm 1.5 ⁱ
5	76.8 \pm 6.8 ^d	7.4 \pm 0.7 ^j
2.5	69.3 \pm 6.2 ^e	4.2 \pm 0.4 ^k
0.8	31.8 \pm 3.2 ^f	3.3 \pm 0.4 ^l
IC ₅₀ (μ M)	1.5	57.5

Different superscripts (a, b, c, d, e, f for L1210 cells and g, h, i, j, k, l for MBL2 cells etc) indicate statistically significant difference ($p < 0.05$) when the data are analyzed by analysis of variance followed by Duncan's multiple range test

Like its counterpart from lily bulbs [5], *Gymnocladus chinensis* trypsin inhibitor fails to inhibit HIV-1 RT and mycelial growth in fungi. In this aspect it differs from its counterpart from the broad bean [37, 38]. It also lacks inhibitory activity toward HIV-1 integrase and SARS coronavirus protease. Unlike broad bean trypsin inhibitor, which manifests antifungal activity, the isolated trypsin inhibitor lacks this activity. This is consistent with the isolation of an antifungal protein but not a trypsin inhibitor when the assay of antifungal activity was used to monitor the purification of antifungal protein from *Gymnocladus chinensis* [35].

An antifungal protein [35] and a trypsin inhibitor [this study] have now been isolated from *Gymnocladus chinensis*. The trypsin inhibitor, in contrast to its counterparts from lily bulbs [40] and *Vigna mungo* [5] inhibits proliferation of tumor cells.

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